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Signalling enzymes: **Bursting with potential**

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A newly identified form of phosphoinositide 3-kinase is regulated by Gβγ subunits and is particularly abundant in phagocytic leukocytes. It is likely to be intimately involved in the process by which inflammatory signals regulate phagocyte activation and is a potential target for new anti-inflammatory drugs.

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Phagocytic white cells, such as neutrophils, are the body's first line of defence. In response to inflammatory signals, such as chemoattractants, they migrate to a site of infection where they engulf and degrade foreign cells. The key player in this process is the NADPH oxidase complex [1], which uses oxygen to rapidly generate vast amounts of the free radical superoxide anion (O_2^-) in what is described as the 'respiratory burst'. Phagocytes also release a variety of proteases in a process of degranulation which parallels the respiratory burst; these proteases are activated by metabolites of superoxide.

The inappropriate activation of phagocytes is implicated in a variety of chronic and acute inflammatory and cardiovascular pathologies characterized by local tissue damage, including asthma, arthritis, reperfusion injury and restenosis. Many pharmaceutical companies are searching for specific antagonists of chemoattractant receptors in the hope that they will control phagocyte migration or activation, but the degree of redundancy between these receptors is unclear and so the efficacy of such an approach remains to be seen. An alternative approach is to target the shared intracellular signalling pathways which are activated by virtually all chemotactic receptors in neutrophils, monocytes, eosinophils and basophils.

Chemoattactants and bacterial peptides activate the phagocyte by binding to G-protein-coupled receptors, typified by the receptors for the bacterial chemotactic peptide f-Met–Leu–Phe or the chemokine, interleukin-8 (IL-8). These activated receptors cause dissociation of heterotrimeric G-proteins to form active Gα–GTP and a Gβγ dimer. Members of the $G\alpha_i$ family of heterotrimeric G proteins are highly expressed in phagocytes and play an important role, as the respiratory burst is attenuated by pertussis toxin, a specific inhibitor of receptor–G_i/G_o interactions.

But what signalling components act downstream of the activated G proteins? The processes of phagocytosis, superoxide production and degranulation require highly organized membrane fusion events, and enzymes that metabolize membrane phospholipids to generate second messengers have therefore attracted particular attention. This is especially true of phosphoinositide 3-kinase (PI 3-kinase), which phosphorylates phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) to yield $PI(3,4,5)P_3$ (reviewed in [2]). PI 3-kinase is intimately involved in phagocyte activation, as drugs that inhibit the enzyme, such as wortmannin or LY294002, completely block the superoxide burst [3,4].

There are, however, multiple forms of PI 3-kinase in cells, and drugs such as wortmannin or LY294002 unfortunately inhibit all forms of the enzyme, to a greater or lesser extent. Given the role of the canonical PI 3-kinases in cell survival and cell growth [5], such drugs may therefore be quite toxic *in vivo*. From a drug discovery standpoint, all that is needed as a promising target is a unique PI 3-kinase that has a novel mode of regulation — and therefore of inhibition — and that is abundant in phagocytes! Stephens *et al.* [6] have now reported the purification and cloning of a Gβγ-stimulated PI 3-kinase which exhibits just these properties.

The first PI 3-kinase to be cloned was found to consist of a regulatory subunit, p85, and a catalytic subunit, p110 (reviewed in [7]). Two isoforms of the catalytic subunit — p110α and p110β — and several regulatory subunits — p55α, p55PIK, p85α and p85β — have been cloned so far. Most is known about the p85 regulatory subunits, which contain several well-known domain types — an SH3 domain, a Bcr-related region and two SH2 domains — consistent with their presumed role in coupling a catalytic p110 subunit to a tyrosine kinase. The SH2 domains bind in a sequence-specific manner to phosphorylated tyrosine residues in activated receptor and non-receptor tyrosine kinases. The p110 catalytic subunit associates with p85 via its amino-terminus, which binds to a region between the SH2 domains of p85 called the inter-SH2 domain [7].

The p85 subunit regulates the p110 subunit in several ways. In unstimulated cells, PI 3-kinase is mainly in the cytosol. Activation of a receptor tyrosine kinase causes recruitment of p85 to the receptor, thereby translocating the p110 catalytic subunit to the plasma membrane, where its substrate, $PI(4,5)P_2$, is found. In addition, the binding of p85 SH2 domains to an appropriately phosphorylated receptor causes a conformational change which is transduced to the catalytic subunit, causing activation.

PI 3-kinase in phagocytes was first described by Traynor-Kaplan *et al.*, in a study of f-Met–Leu–Phe-stimulated neutrophils [8]. Several observations suggested that this enzyme was distinct from the canonical p85–p110 form. First, the phagocyte PI 3-kinase was stimulated by agonists, such as ATP, that activate G-protein-coupled receptors rather than receptor tyrosine kinases. Second, the activity in phagocytes was inhibited by pertussis toxin but was insensitive to the kinase inhibitors genistein or staurosporine [9], and exhibited a reduced sensitivity to wortmannin [10]. Third, the phagocyte PI 3-kinase could be stimulated by Gβγ subunits but not by tyrosine-phosphorylated peptides that activate p85–p110, and could not be found in anti-p85 immunoprecipitates [10]. Finally, compared to the slow and sustained activation induced by factors that induce the p85–p110 enzyme, the increase in $PI(3,4,5)P_3$ levels in response to f-Met–Leu–Phe or ATP was rapid and transient, suggesting distinct modes of regulation [9,11]. These observations suggested the existence in phagocytes of a unique form of PI 3-kinase, and the race was on to clone the new enzyme.

In 1995, Stoyanov *et al.* [12] cloned a cDNA for a novel p110-related enzyme by homology to conserved regions of p110α and p110β. This new PI 3-kinase, called p110γ, could be activated by Gβγ but was also stimulated by Gα–GTP, in contrast to reports on the partially purified enzyme from neutrophils [10]. Furthermore, the activation by Gβγ was very weak compared to the partially purified preparations [10]. Clearly something was missing. The missing piece to this puzzle has now dropped into place, with the purification and cloning of a Gβγ-stimulated PI 3-kinase activity from pig neutrophils, a tightly-associated dimer of two subunits, p101 and p120 [6]. Cloning of these subunits showed that p120, which binds wortmannin, is highly homologous to the p110γ catalytic subunit [12] — so similar that it has been renamed p110γ — but significant Gβγ stimulation requires the associated regulatory subunit, p101 [6]. The small activation of isolated p110γ by Gβγ observed previously [12] probably reflects effects of the detergents used to store Gβγ on the lipid micelle mix used to assay PI 3-kinase activity.

The p110γ subunit is clearly distinct from p110α and p110β. These catalytic subunits are most similar in their carboxy-terminal kinase domain (homology region 1), but there is also significant sequence overlap in the PI 3-kinase-specific homology regions 2 and 3, the function of which remains unclear. All three isoforms contain a Ras-binding domain [13], and preliminary data indicate that co-expression of activated Ras can augment the Gβγ stimulation of p101–p110γ in COS cells (L. Stephens, personal communication). As expected, p110γ does not show any homology to the amino-terminal p85-binding site of p110α/p110β and does not bind to p85 [6,12].

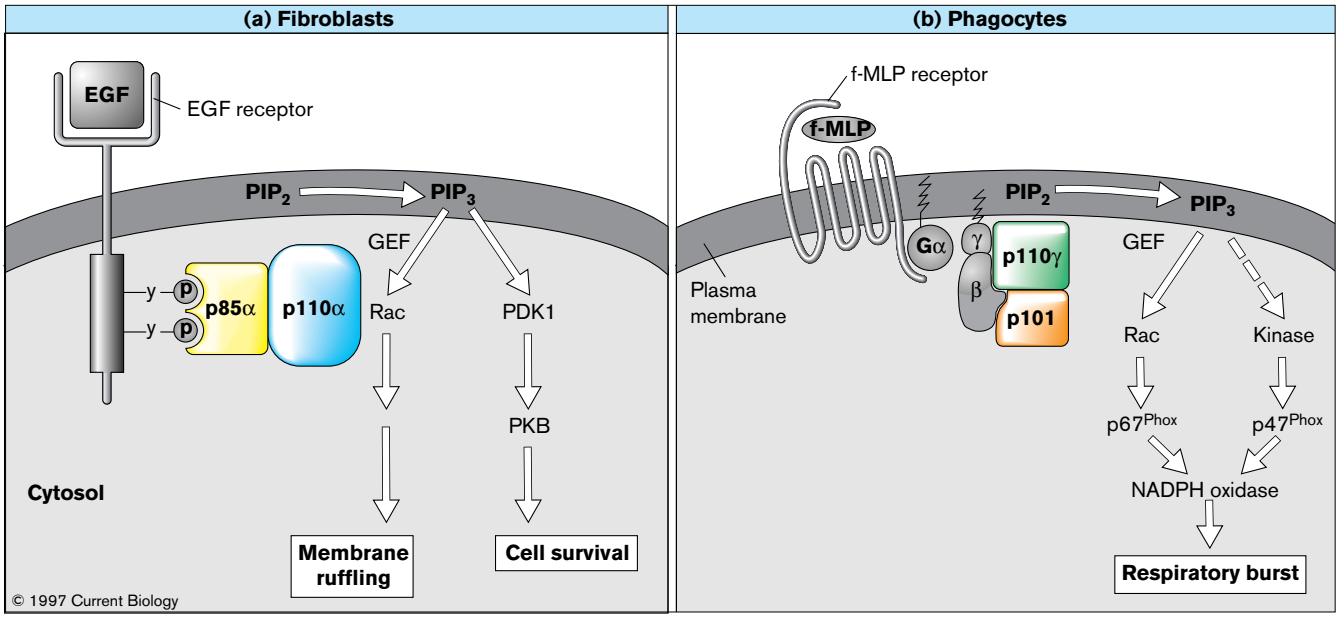
The p101 subunit is something of a 'black box' in structural terms, as it does not show any homology or structural similarity to any proteins in the database [6]. By analogy with the canonical p85–p110 enzyme, there are likely to be discrete regions on both p101 and p110γ which mediate their interactions, but it is not yet clear whether the Gβγ subunits bind to p101 or p110γ (see below). Stoyanov *et al.* [12] reported that the amino-terminus of p110γ shows some homology to the Ras-GAP pleckstrin homology (PH) domain, and speculated that this region could be the binding site for Gβγ. However, this homology was very weak and was not detected by others [6]. Furthermore, it seems increasingly unlikely that PH domains function as Gβγ-binding modules.

In considering the regulation of p101–p110γ by Gβγ, we can learn from other Gβγ-regulated enzymes. The role played by Gβγ subunits in directly binding to and regulating the activity and subcellular distribution of signalling proteins is now generally appreciated. In addition to the p101–p110γ enzyme described by Stephens *et al.* [6], other effector molecules known to be regulated by direct binding of Gβγ include the GIRK1, GIRK2 and GIRK4 subunits of inwardly rectifying G-protein regulated K^+ channels, adenylyl cyclase (AC) isoforms II and IV, and phosphatidylinositol-specific phospholipase C (PLC) β1, β2 and β3 isoforms [14].

The prototypical Gβγ-regulated effector enzyme is the G-protein-coupled receptor kinase 2 (GRK2), which specifically binds to and phosphorylates agonist-occupied G-protein-coupled receptors, thereby promoting receptor desensitization. Upon receptor-mediated dissociation of a heterotrimeric G-protein into Gα–GTP and Gβγ-subunits, the released Gβγ-subunits, which remain attached to the plasma membrane, bind to GRK2, causing it to translocate to the site of the activated receptor at the plasma membrane [15].

Mutational analysis of GRK2 has shown that its Gβγ-binding site includes the carboxyl-terminal region of a PH domain, which has been found in many other signalling proteins and initially prompted the suggestion that PH domains may function as general Gβγ-binding domains [16]. This is most likely not the case, as other PH domains have a 50–100-fold lower affinity for Gβγ-subunits than has GRK2 [17], and high-affinity binding of GRK2 to Gβγ subunits also requires a 30 amino-acid carboxy-terminal sequence outside of the PH domain [16]. However, a Q^{613}XXER sequence motif present within the carboxy-terminal section of the GRK2 PH domain is also found in several other Gβγ-regulated enzymes, and the Gβγ-stimulated activity of AC-II, PLCβ3 and GRK2 can be reduced in a concentration-dependent manner by a QXXER-containing peptide derived from the second cytoplasmic domain of AC-II [18].

Figure 1



Regulation of PI 3-kinases by receptor tyrosine kinases and G-protein coupled receptors. The regulation of p101–p110γ and p85–p110α and their potential downstream targets are indicated schematically. Both PI 3-kinases have a similar, sequential 'translocation–activation' mechanism, but they use distinct allosteric regulators to achieve this end. **(a)** Using fibroblasts as an example, p85–p110α is recruited via the SH2 domain of p85 to the activated, phosphorylated epidermal growth factor (EGF) receptor. This has the effect of translocating the catalytic p110α subunit to the plasma membrane and activating it by inducing a conformational change. (GEF, Rac GDP/GTP exchange factor.) **(b)** In phagocytes, recruitment of p101–p110γ to the membrane and activation are achieved by binding Gβγ subunits released from trimeric G proteins after f-Met–Leu–Phe (fMLP) receptor-mediated activation; the Gβγ -binding site and, therefore, mechanism of activation remains to be defined.

Although p101 confers Gβγ-sensitivity upon the p101–p110γ dimer, it is unclear whether one or both of the subunits binds Gβγ *in vivo* or *in vitro* [6]. Neither subunit contains a QXXER motif, although related sequences include Q^{14}HALER and Q^{818}DER in p101 and Q^{922}AAVER in p110γ [6]. So it is possible that binding of Gβγ subunits to p101–p110γ may involve interactions distinct from those described for other effector enzymes. It is also possible that p101–p110γ, together with phosducin, phosducin-like protein and PLC-β2, all of which bind Gβγ subunits in the absence of QXXER sequences [19,20], uses a distinct type of Gβγ-binding site.

Without a clear understanding of the site of interaction with Gβγ, the mechanism of activation of p110γ by p101 remains unclear. Interestingly, in the absence of Gβγ, the specific activity of p110γ is lower in a p101–p110γ complex than when it is a free monomer [6]. This is reminiscent of the p85–p110α complex, which has a lower specific activity than free p110α. In the case of p85–p110α, this is due in part to the ability of p110α to phosphorylate p85 in the inter-SH2 domain (reviewed in [7]). It is not known if a similar event occurs with p101–p110γ, nor whether the binding of phospho-tyrosine peptides or Gβγ counteracts this negative regulation.

From the available data, it appears that *in vitro* the regulatory subunit of PI 3-kinase represses the basal activity of the catalytic subunit, but renders it activatable by the appropriate allosteric regulator.

As the p101–p110γ complex is cytosolic, yet its substrate is a membrane phospholipid, it must translocate to the plasma membrane. Presumably, Gβγ-subunits released upon G-protein activation bind to the p101–p110γ complex, thereby facilitating its translocation to the plasma membrane in a manner dependent on receptor activation. Once bound to Gβγ, p101–p110γ is presumably activated in the manner described by Stephens *et al.* [6]. In this respect, the mobilization of PI 3-kinase activity closely resembles the process by which GRK2 phosphorylates agonist-occupied G-protein-coupled receptors — in both cases, Gβγ facilitates the translocation of the enzyme to its membrane-bound substrate and also its activation [15]. It also parallels the translocation/activation mechanism of p85–p110α complexes by receptor tyrosine kinases, and shows that, with respect to regulation of PI 3-kinase activity, G-protein coupled receptors and receptor tyrosine kinases can use the same activation mechanism — sequential enzyme translocation and activation — by using distinct protein-protein interaction domains — SH2 domains for receptor tyrosine

kinases and Gβγ-binding domains for G-protein-coupled receptors (Figure 1).

What are the downstream targets of p101–p110γ in phagocytes? In fibroblasts, PI 3-kinases act upstream of members of the Rac family of monomeric GTPases, which regulate the actin cytoskeleton so as to cause membrane ruffles [21]. Rac is also involved in assembly of the NADPH oxidase [1] by binding to one of the cytosolic components of the enzyme complex, $p67^{phox}$ (Figure 1). These observations are consistent with a model in which activation of p101–p110γ in turn promotes activation of Rac, perhaps by stimulating a Rac GDP/GTP exchange factor, and thereby assembly of the oxidase complex.

Recent studies have indicated that the canonical PI 3-kinases act upstream of a kinase cascade which regulates the activity of PKB/c-Akt, a serine/threonine kinase implicated in promoting cell survival [5]. Although it is possible that PKB may also be a downstream target of p101–p110γ in phagocytes, an alternative possibility is that $PI(3,4,5)P_3$ may activate another kinase which serves to phosphorylate $p47^{phox}$, the other cytosolic NADPH oxidase component known to be phosphorylated in a PI 3-kinase-dependent manner [22] (Figure 1). Elucidation of the precise role of p101–p110γ in these processes may be facilitated by the generation of suitable gene 'knockout' or transgenic mice.

The PI 3-kinase subunits p101 and p110γ appear to be particularly abundant in leukocytes (A. Eguinoa, P. Hawkins and L. Stephens, personal communication), in contrast to the canonical forms of PI 3-kinase, which have a broader distribution. It is already known that PI 3-kinase catalyses a rate-limiting step in phagocyte activation [3,4], and the novel properties discussed above make p101–p110γ an attractive target for attempts to discover new anti-inflammatory drugs that work by selectively inhibiting PI 3-kinase-dependent phagocyte activation.

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